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Virus reactivation in pigs latently infected with a thymidine kinase negative vaccine strain of pseudorabies virus

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Summary. Attenuated, gene-deletion mutants of pseudorabies virus (PRV) were tested for their ability to establish a reactivatable latent infection in pigs. The viruses (designated A, B, and C) were from each of three vaccines commercially available in the United States. Viruses A and C were similar in that they had genetically engineered gene deletions for thymidine kinase (TK) and glycoprotein X (gX); however, they had been prepared from genetically different parental strains. Virus B was TK positive, but had a naturally occurring gene deletion for glycoprotein I (gI). Four pigs were exposed oronasally to each of the viruses, and 10 weeks later they were treated with dexamethasone in an attempt to induce virus reactivation. All of the viruses replicated after initial exposure as evidenced by virus isolation from nasal swabs and the pigs' immune responses. Virus reactivation was subsequently induced by dexamethasone treatment in two of four pigs exposed to virus A. Notably, both pigs remained free of serum antibody for gX. Restriction endonuclease analysis and tests for TK activity and the presence of gX indicated that reactivated virus was similar, if not identical, to virus A used to establish latent infection. Virus shedding after dexamethasone treatment was not identified for either of the other pigs exposed to virus A nor for any of the pigs exposed to viruses B or C. The results indicated that attenuated, TK-negative PRV can establish a reactivatable, latent infection in pigs.

Introduction

In general, the vaccination of pigs with attenuated pseudorabies virus (PRV), family *Herpesviridae*, subfamily *Alphaherpesvirinae*, prevents severe clinical signs and death that may otherwise follow exposure to virulent PRV [7, 14]. Vaccination does not, however, prevent either acute or latent infection with virulent PRV [8, 21, 30]. As a consequence, vaccinated pigs, as well as nonvaccinated

pigs, that survive infection with virulent PRV can become virus carriers and a source of virulent PRV following virus reactivation.

Until recently, there has been little more than academic interest in the possibility that attenuated PRV can also become latent since the potential spread of such virus was generally considered of little or no clinical or economic significance. The situation has now changed, however, with the initiation of pseudorabies (PR) eradication programs in the United States [26] and Europe [13, 27–29]. The success of these programs may depend in large part on the use of deletion mutant vaccines and their companion diagnostic tests to identify pigs exposed to virulent PRV regardless of their vaccination status. Differentiation is based on the principle that serum antibody to a virus-coded protein for which the deletion mutant is defective is only present if the pig has been exposed to virulent virus [31, 33]. Implicit in this procedure for differentiation is the assumption that virulent deletion mutants for the protein in question do not occur naturally. Moreover, it is equally important that they not be created through recombination of vaccine and virulent virus. While it is clear that virulent virus can establish latent infection in vaccinated pigs, it is not known whether the same pigs are also latently infected with the vaccine virus. If so, simultaneous reactivation of both populations could result in recombinant strains of virus that are virulent as well as defective for the protein used for differentiation.

The purpose of the study reported here was to investigate the ability of three commercially available PR vaccine viruses to establish a reactivatable state of latent infection in pigs. In an attempt to increase the likelihood of latency, each of the viruses was administered oronasally. The fact that viruses of two of the three vaccines tested were thymidine kinase (TK) negative provided an opportunity to also address the fundamental question of whether the lack of TK activity effectively precludes either latency or reactivation of PRV as suggested by previous studies [6, 12, 16, 32].

Materials and methods

Experimental design

Pigs were randomly assigned to each of three treatment groups (four pigs/group) and one control group (three pigs). Thereafter, each of the groups was kept in a separate isolation room. Each of the treatment groups was exposed to a different, commercially available, deletion-mutant, PR vaccine virus that had been serially passed twice in cell cultures in our laboratory. Each pig within a treatment group was exposed oronasally to $2 \times 10^{6.0}$ plaque forming units (PFU) of the appropriate vaccine virus. Pigs of the control group were not exposed to virus, but were otherwise treated similarly throughout the experiment. Ten weeks later, all pigs (virus-exposed and controls) were treated with dexamethasone to determine if any of the vaccine viruses had established a reactivatable latent infection. Body temperatures were taken and nasal and oropharyngeal swabs were collected immediately before and for 10 days after pigs were exposed to virus, and again during a similar interval when the pigs were treated with dexamethasone. A blood sample was collected from each pig immediately before it was exposed to vaccine virus (week 0) and 4, 10 (i.e., immediately

before the start of dexamethasone treatment), and 13 weeks later. Body temperatures were taken for, and swabs and blood samples were collected from, pigs of the control group at the same times. Nasal and oropharyngeal swabs were tested for the presence and titer of PRV and sera were tested for virus neutralizing (VN) and precipitating antibodies for PRV. Some of the virus isolates were further characterized and compared to the original vaccine viruses, and selected sera were tested for antibodies to viral glycoprotein X (gX) [15].

Pigs

Crossbred pigs that were 7 weeks old and weighed about 14.5 kg (on average) at the start of the experiment were from a commercial swine herd. The herd was free of PRV infection, and none of the pigs had been vaccinated for PR.

Cell cultures

An established porcine kidney (PK-15) cell line was used for propagation of vaccine viruses, for plaque assays, and for VN tests. A TK-deficient variant of the established Madin-Darby bovine kidney (MDBK) cell line [1] (provided by W.C. Lawrence, University of Pennsylvania) was used to test for viral TK activity. A bovine embryonic spleen (BESp) cell strain [17] was used to test nasal and oropharyngeal swabs for infectious virus. These cells were found previously to be at least as susceptible as PK-15 cells to PRV and were easier than PK-15 cells to maintain as healthy monolayers for the relatively long interval, at least 5 days, used to test swabs for infectious PRV. All types of cells were propagated in growth medium that consisted of Eagle minimal essential medium (EMEM) supplemented with 0.5% lactalbumin hydrolysate, gentamicin sulfate (50 μg/ml), and either 5% fetal bovine serum (FBS) for PK-15 cells or 10% FBS for MDBK and BESp cells. When PK-15 cells were used to propagate vaccine viruses, they were infected as confluent monolayers and thereafter maintained in growth medium free of serum. When BESp cells were used to test swabs for infectious virus, the growth medium was supplemented with additional gentamicin (500 μg/ml) and other antibiotics; namely penicillin (25 units/ml), streptomycin (25 mg/ml), neomycin sulfate (25 μg/ml), bacitracin sulfate (0.25 units/ml), and amphotericin B (2 μg/ml).

Viruses

Viruses from each of three commercial, deletion-mutant vaccines for PR (identified in this report as viruses A, B, and C) were serially passaged twice in an established PK-15 cell line to prepare the stocks of virus used in the experiment. Virus A had genetically engineered gene deletions for gX and TK. Virus B had a naturally occurring gene deletion for viral glycoprotein I (gI). Virus C was similar to virus A in that it had genetically engineered gene deletions for gX and TK. However, it had been developed in a different laboratory from a different parental strain of PRV.

The virulent Indiana-F strain of PRV was used to test sera for VN antibody and as a wild type (TK- and gX-positive) virus control in tests for TK activity and the presence of gX.

Virus neutralization

Sera were tested for VN activity with the aid of an automated system (Pro/pette, Cetus, Emeryville, CA). Sera were heat inactivated (56 °C, 30 min) and then diluted in 2-fold increments in EMEM in 96-well cell culture plates so that each of eight wells contained 50 μl of each serum dilution. Approximately 100 PFU of virus in 50 μl of EMEM were added to each well and plates were incubated at 37 °C for 24 h [2] in a humid atmosphere

of 5% CO_2. Approximately 5000 PK-15 cells in 100 µl of growth medium containing 10% FBS were added to each well, and plates were again incubated at 37 °C in a humid atmosphere of 5% CO_2. Plates were examined daily for cytopathic effects and the VN titer was expressed as the maximal initial dilution of serum (i.e., before adding virus and cells) that protected cells in at least four of the eight wells.

Radioimmunoprecipitation

Methods for preparation of [^{35}S]methionine- and [^{14}C]glucosamine-labelled viral proteins, immunoprecipitation, and polyacrylamide gel electrophoresis were as described elsewhere [18, 19].

Detection of viral glycoprotein X and homologous antibody

Culture medium collected from a cell culture infected with the PRV in question was tested by radioimmunoprecipitation (RIP) for the presence of gX. Viral glycoproteins were radiolabeled with [^{14}C]glucosamine and precipitated with a pig anti-PRV serum known to have a high titer of antibody for gX. The test was interpreted by the presence or absence of a glycoprotein of appropriate molecular weight (approximately 90 k) in conjunction with its presence and absence, respectively, in culture medium collected from known gX-positive and gX-negative strains of PRV run in parallel. In addition, medium collected from the strain in question was used as antigen to coat wells of a 96-well plate. The wells were subsequently tested for gX by enzyme-linked immunoassay (ELISA) using a commercially available monoclonal antibody for gX (HerdChek, AgriTech Systems, Inc., Portland, ME). Known gX-positive and gX-negative strains of PRV were included as controls.

Sera were tested for antibody to gX with a commercially available ELISA kit in the manner recommended by the manufacturer (HerdChek, AgriTech Systems, Inc., Portland, ME).

Plaque assay

Virus was diluted in 10-fold increments. Nutrient medium was aspirated from confluent monolayers of PK-15 cells in 28 cm^2 petri dishes and then 1 ml of undiluted virus and each 10-fold dilution was added to each of two cultures. The inoculum was uniformly distributed over the culture surface and virus was adsorbed for 1 h at 37 °C in a humid atmosphere of 5% CO_2 with occasional redistribution of inoculum. The inoculum was then aspirated, and the cultures were each overlaid with 4 ml of growth medium containing 0.5% agar at 45 °C. After solidification of the agar at 22 °C, culture plates were inverted and incubated at 37 °C for 2 to 3 days in a humid atmosphere of 5% CO_2. Plaques were counted and the virus titer expressed as PFU/ml.

Thymidine kinase activity

Viruses were tested for TK activity essentially as described by others [25]. Briefly, confluent monolayers of TK-deficient MDBK cells in 60 mm petri dishes were infected with the virus in question at a multiplicity of infection that allowed discrete plaques to develop under agar overlay (0.7% agar in growth medium). When plaques became macroscopically obvious at about 48 h after infection, the agar overlay was removed, and each culture was incubated at 37 °C for 5 h in the presence of 1 ml of growth medium containing 0.5 µCi of [2-^{14}C]-thymidine (54 mCi/mM; Dupont). The radioactive medium was then removed, and cultures were rinsed twice with EMEM to remove residual unincorporated radiolabel. Cultures were fixed in 70% acetone, and the sides of each petri dish were removed. The fixed cultures were used to expose X-ray film (X-Omat AR, Eastman Kodak). An exposed (dark) periphery

of virus-induced plaques indicated incorporation of the radioisotope and thus virus TK activity.

Nasal and oropharyngeal swabs

Cotton-tipped swabs were applied to intranasal and oropharyngeal surfaces of each pig at each of the selected times by the following procedure. A nasal swab was inserted about 5 cm into each nostril (with precaution to minimize damage to the nasal mucosa), and an oropharyngeal swab was "rubbed" over most of the surface of the tonsil and soft palate. Each swab was immediately submerged in 2 ml of cold (about 4 °C) EMEM containing gentamicin sulfate (500 μg/ml), penicillin (25 units/ml), streptomycin (25 mg/ml), neomycin sulfate (25 μg/ml), bacitracin sulfate (0.25 units/ml), and amphotercin B (2 μg/ml) in a 13 by 100 mm screw-capped tube. The tubes were kept on ice during transport from the animal isolation facility to the laboratory and then frozen at −20 °C. To test for virus, the tube contents were thawed and then mixed vigorously, and an aliquot (0.2 ml) of the liquid was added to the medium of a confluent monolayer (2 cm^2) of BESp cells. The cultures were thereafter examined daily for PRV-induced cytopathic effects. Samples found to contain virus were subsequently titrated for infectivity by plaque assay.

Dexamethasone treatment

In an attempt to reactivate latent PRV, all pigs (virus-exposed and controls) were treated with a 5-day series of dexamethasone injections [5, 20] beginning 10 weeks after pigs were first exposed to virus. The dosage of dexamethasone/0.45 kg of body weight and the routes of injections were: 1.25 mg given intravenously (IV) and 0.25 mg given intramuscularly (IM) in the morning and 0.5 mg given IM in the afternoon of the 1st day; 0.25 mg given IV and 0.25 mg given IM in the morning and 0.5 mg given IM in the afternoon for the next 4 days.

Restriction endonuclease analysis

The procedures for restriction endonuclease analysis of viral genomes were as previously described [22].

Results

Virus was isolated from nasal swabs of all pigs exposed oronasally to viruses A and B, and from two of the four pigs exposed oronasally to virus C. The duration of shedding ranged from 4 to 7 days for pigs exposed to virus A, 1 to 5 days for pigs exposed to virus B, and was 2 and 4 days for the two pigs that shed virus after exposure to virus C. The amount of virus shed during these intervals was greatest for pigs exposed to virus A (Table 1). In contrast to the frequent isolation of virus from nasal swabs, virus was isolated from only one of the oropharyngeal swabs collected at the same times (pig 3, fifth day postexposure). Virus was not isolated from either nasal or oropharyngeal swabs collected at the same times from the other two pigs exposed to virus C nor from non-exposed (control) pigs. Detectable virus shedding ceased for all pigs by the ninth day after oronasal exposure.

Dexamethasone treatment at week 10 resulted in virus reactivation and shedding for pigs 1 and 3 that had been exposed to virus A. Pig 1 shed virus in nasal secretions at least 7 consecutive days at titers of up to $10^{4.8}$ PFU/ml. Pig 3 shed virus for a single day (Table 2).

Table 1. Isolation of vaccine strains of pseudorabies virus (PRV) from nasal swabs of pigs exposed oronasally[a]

Virus	Pig no.	Days after oronasal exposure to vaccine viruses										
		0	1	2	3	4	5	6	7	8	9	10
A	1	–	–	<1.0[b]	<1.0	2.0	<1.0	–	–	–	–	–
	2	–	–	<1.0	3.1	3.2	2.8	2.1	–	–	–	–
	3	–	–	2.3	2.4	3.1	3.4	2.8	–	–	–	–
	4	–	–	2.5	3.0	2.0	2.5	2.2	<1.0	<1.0	–	–
B	5	–	–	–	–	–	<1.0	–	–	–	–	–
	6	–	–	<1.0	–	1.4	<1.0	–	<1.0	–	–	–
	7	–	–	–	<1.0	<1.0	<1.0	<1.0	–	<1.0	–	–
	8	–	–	–	<1.0	<1.0	<1.0	–	–	–	–	–
C	9	–	–	–	–	–	<1.0	<1.0	–	–	–	–
	10	–	<1.0	–	<1.0	<1.0	<1.0	–	–	–	–	–
	11	–	–	–	–	–	–	–	–	–	–	–
	12	–	–	–	–	–	–	–	–	–	–	–

[a] Oropharyngeal swabs were collected at the same times; however, PRV was isolated only from the swab collected on the 5th day from pig 3. Virus was not isolated from any of the nasal or oropharyngeal swabs collected at the same times from the nonexposed control pigs 13, 14, and 15

[b] Titers expressed in $\log_{10}$ plaque forming units/ml (swabs were submerged in 2 ml of transport medium for testing). *<1* Virus isolated, but at a titer of $<10^1$. – No virus isolated

Table 2. Isolation of a vaccine strain of pseudorabies virus (PRV) from nasal swabs of pigs treated with dexamethasone to reactivate latent virus[a]

Virus	Pig no.	Days after start of dexamethasone treatment										
		0	1	2	3	4	5	6	7	8	9	10
A	1	–	–	–	–	1.2[b]	<1.0	2.9	4.3	4.8	2.9	1.2
	3	–	–	–	–	–	–	–	<1.0	–	–	–

[a] PRV also was isolated from oropharyngeal swabs collected on days 4, 7, and 8 from pig 1 (titers were all <1.0). It was not isolated after dexamethasone treatment from any of the nasal or oropharyngeal swabs collected from any of the other principal or control pigs

[b] Titers are expressed as $\log_{10}$ plaque forming units/ml (swabs were submerged in 2 ml of transport medium for testing). *<1* Virus isolated, but at a titer of $<10^1$. – No virus isolated

None of the pigs had any clinical signs of PR and there were no marked increases in body temperatures after either oronasal exposure to virus or subsequent treatment with dexamethasone.

Table 3. Virus neutralization (VN) titers of sera collected from pigs after oronasal exposure to vaccine strains of pseudorabies virus (PRV) and subsequent dexamethasone treatment

Vaccine	Pig no.	Weeks after oronasal exposure			
		0	4	10[a]	13
A	1	<2	32	32	2048
	2	<2	16	32	64
	3	<2	512	128	128
	4	<2	128	128	128
B	5	<2	16	64	64
	6	<2	8	32	16
	7	<2	32	16	32
	8	<2	4	16	16
C	9	<2	2	8	8
	10	<2	4	8	8
	11	<2	<2	<2	<2
	12	<2	32	32	16

Sera from blood collected at the same times from control pigs 13, 14, and 15 were all free (titers = <2) of VN antibody for PRV

[a] A 5-day series of dexamethasone injections was started just after blood samples were collected at week 10

Most of the sera collected from pigs after exposure to viruses A, B, or C had both VN antibodies (Table 3) and precipitating antibodies (Fig. 1) for PRV. The only exception was sera from pig 11 exposed to virus C. In general, VN titers either remained the same or varied no more than 4-fold between the fourth and thirteenth weeks postexposure. The exception was pig 1 that had a 64-fold increase in VN titer between weeks 10 and 13; presumably as a result of virus reactivation. In contrast, precipitating activity of most sera increased between the fourth and tenth week postexposure (Fig. 1). There also was a marked increase in serum precipitating activity between weeks 10 and 13 for pig 1 and a lesser increase during the same interval for pigs 2, 7, and 8, and perhaps for pigs 5 and 6. For the other pigs, there was no detectable change. Notably, there was no change in sera precipitating activity between weeks 10 and 13 for pig 3 even though virus reactivation was confirmed for this pig by virus isolation.

To determine if the reactivated virus isolated from pigs 1 and 3 was similar or identical to virus A to which these pigs had been exposed initially, aliquots of reactivated virus were compared to virus A by restriction endonuclease analysis. Reactivated virus also was tested for TK activity and synthesis of gX. Collectively, these tests indicated that few, if any, genomic changes had occurred in virus A either during latency or subsequent reactivation. Restriction patterns

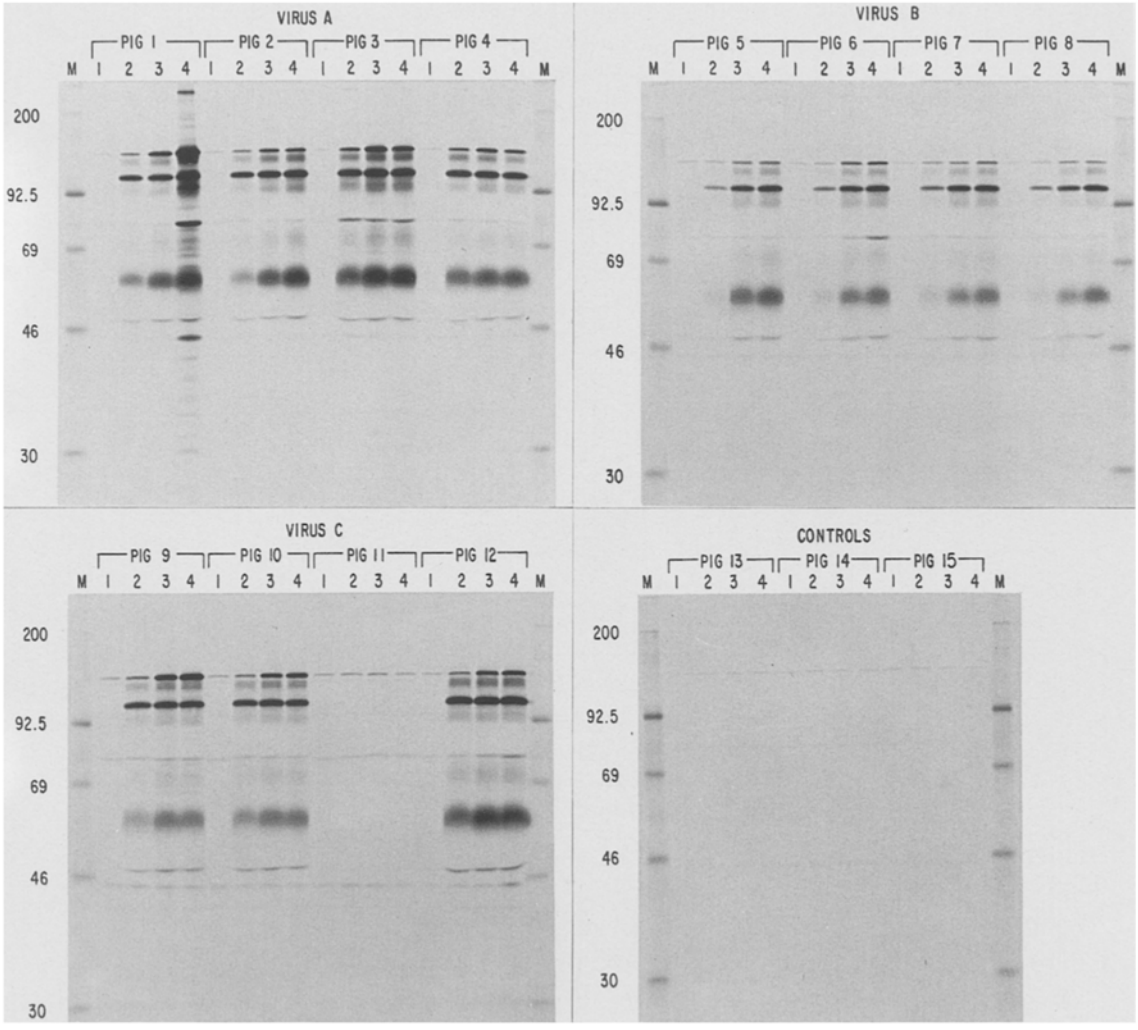

Fig. 1. Radioimmunoprecipitation of [^{35}S]methionine-labelled pseudorabies virus (PRV) proteins (infected-cell lysate) with sera collected from pigs exposed to attenuated PRV (A, B, C) and from non-exposed pigs (controls). Sera were collected immediately before pigs were exposed to virus (*1*), and 4 (*2*), 10 (*3*), and 13 (*4*) weeks later. *M* Molecular weight markers (values $\times 10^3$). Immediately after sera were collected at 10 weeks, all pigs were started on a 5-day series of dexamethasone injections. Note the marked increase in serum precipitating activity of pig 1 between weeks 10 and 13 and a lesser increase during the same interval for pigs 2, 7, and 8 and perhaps for pigs 5 and 6

of reactivated virus were the same as those of virus A and different markedly from those of viruses B and C (Fig. 2). Reactivated virus also was like TK-negative virus A in that it did not incorporate [^{14}C]thymidine during replication in cell culture as did TK-positive wild-type (strain Ind-F) PRV and TK-positive virus B (Fig. 3). The absence of gX synthetic activity of reactivated virus was indicated by several observations. First, no gX was detected by RIP in the medium of cell cultures infected with reactivated virus. Second, no gX was

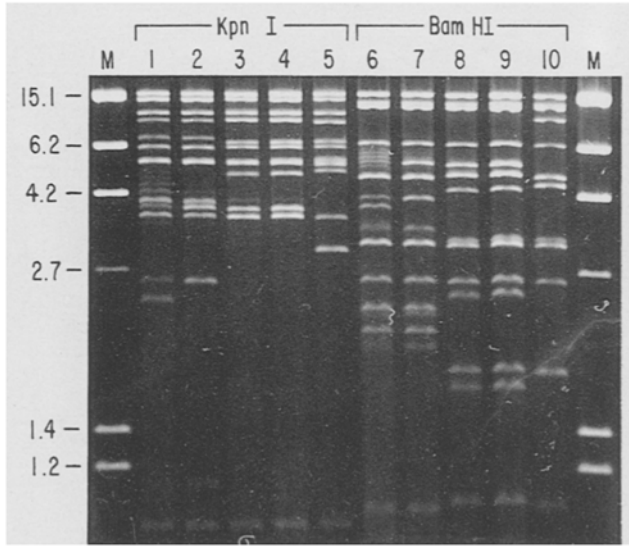

Fig. 2. Restriction endonuclease analysis of pseudorabies viral DNA digested with either Kpn I (*1–5*) or Bam HI (*6–10*). Digests are of stock virus B (*1* and *6*), virus isolated from pig 7 on the 4th day after it was exposed oronasally to virus B (*2* and *7*), stock virus A (*3* and *8*), reactivated virus isolated from pig 3 on the 7th day after the start of dexamethasone injections (*4* and *9*), and stock virus C (*5* and *10*). *M* Digests of bacteriophage lambda DNA with Hind III are included as MW markers (values $\times 10^6$). The restriction patterns of reactivated virus isolated from pig 1 (not illustrated) were the same as those of stock virus A and virus reactivated from pig 3

detected when medium from cell cultures infected with reactivated virus was used as antigen in an ELISA test and subsequently reacted with a monoclonal antibody for gX. Third, no antibody for gX was detected by blocking ELISA in sera collected from pigs 1 and 3 before and after virus reactivation.

Discussion

The results of this study indicate that an attenuated, TK-negative vaccine strain of PRV can establish a reactivatable latent infection in pigs. The likelihood of this event occurring under field conditions is difficult to predict; and variables such as route and magnitude of exposure as well as innate host resistance may play important roles. However, regardless of the circumstances that might contribute to virus latency and reactivation, it is clear that for pigs the lack of TK activity does not preclude either. In fact, a comparison of results with TK-negative virus A and TK-positive virus B suggests that the amount of virus replication (Table 1) may be a more important determinant for these events. If so, the degree of virus attenuation, regardless of how it is achieved, may be the best predictor for the likelihood of reactivation.

Despite the lack of definitive evidence for reactivation of virus from pigs other than those exposed to virus A, the possibility that pigs exposed to the

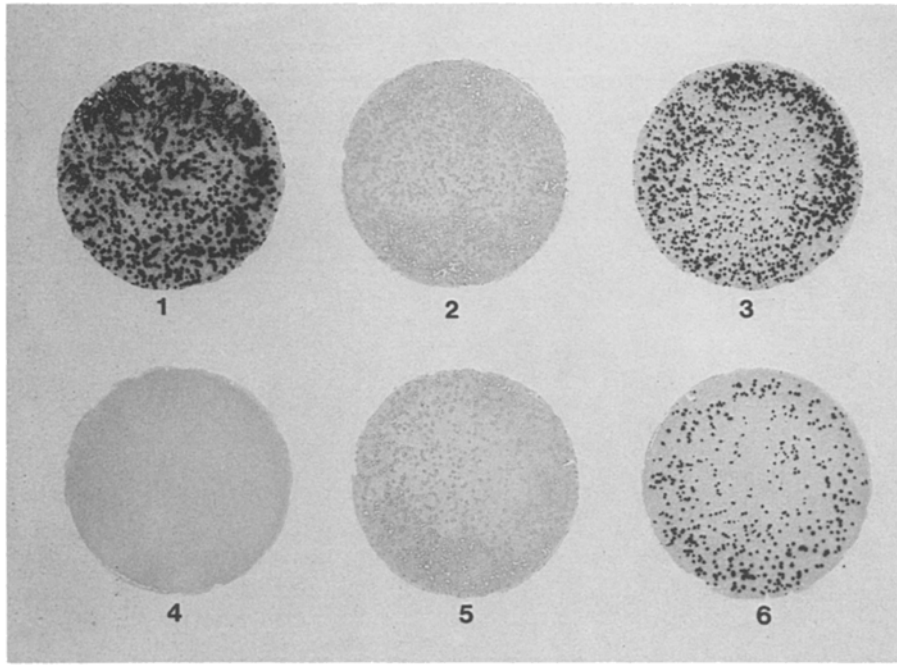

Fig. 3. Tests for viral thymidine kinase (TK) activity. Pseudorabies virus (PRV) was propagated in a TK-deficient cell line in the presence of [2-^{14}C]thymidine as described by Tenser et al. [25]. Reactions are illustrated for: *1* virulent, TK-positive PRV; *2* attenuated, TK-negative virus A; *3* attenuated, TK-positive virus B; *4* noninfected cell culture; *5* reactivated virus isolated from pig 3 on the 7th day after the start of dexamethasone injections; and *6* virus isolated from pig 7 on the 4th day after it was exposed oronasally to virus B

other viruses were also latently infected was not excluded. Notably, others [11] have used in situ hybridization to identify PRV genomes in trigeminal ganglia of pigs killed 8 weeks after they were exposed to an attenuated, TK-positive, recombinant virus.

Based on the findings presented here (Table 2) and those recently reported for cattle infected with a TK-negative mutant of bovine herpes virus type 1 [34], it is possible that TK activity has no direct role in reactivation of alpha herpes viruses in their natural hosts. The situation may differ in animal models, however, in that latent infections with TK-negative mutants of herpes simples virus type 1 have been established in both rabbits [3] and mice [4, 23, 24], but subsequent reactivation has been observed only in rabbits [3].

Naturally occurring reactivation of an attenuated vaccine strain of PRV would have the potential to complicate efforts for PR eradication in any one of several ways. If virus subsequently spread to nonvaccinated pigs, they might be assumed to have been infected with virulent "wild type" virus. If it spread to pigs previously vaccinated with a deletion mutant virus, it could cause seroconversion for the diagnostic antigen; unless, by chance, both viruses had the same gene deletion(s). However, it is unlikely that either of these scenarios

would pose a major problem because it is unlikely that attenuated virus would spread extensively by pig-to-pig transmission. Moreover, if the virus in question were a gene-deletion mutant, its dissemination to nonvaccinated pigs could be recognized by the appropriate diagnostic test. Of greater theoretical consequence is the possibility that through dual latency and reactivation of vaccine and virulent viruses, a virulent, but gene-deleted, recombinant could emerge and spread to susceptible pigs. If so, the differential diagnostic tests used to confirm or deny exposure to virulent PRV would be invalid. Test results could actually contribute to the spread of virulent PRV through the approved movement of presumably vaccinated pigs that were instead carriers of virulent virus. The fact that virus recombination can occur in an infected animal has been established for both sheep [10] and pigs [9] under experimental conditions by simultaneously exposing them to two strains of PRV. Moreover, the amount and duration of virus replication in pig 1 (Table 2) of this study suggest that there would have been ample opportunity for recombination to occur if virulent virus had been reactivated simultaneously or if the pig had been exposed initially to virulent virus during the reactivation interval. While one may argue logically that there is a low probability for the aforementioned series of events leading to recombination, it should be kept in mind that millions of pigs are vaccinated annually for PRV and that many of these are subsequently exposed to, and become latently infected with, virulent virus.

On the basis of virus isolation and immune responses, it seems likely that vaccine viruses replicated in at least 11 of the 12 exposed pigs. It may have replicated in the remaining pig (pig 11) as well, but at a level that was not detected by either virus isolation or a primary immune response. Otherwise, the most obvious consideration is that pig 11 was somehow missed during initial oronasal exposure to virus. While this possibility cannot be excluded, it is difficult to accept with only four pigs in the treatment group. Another consideration is that the infectious dose of this strain of virus for pigs exposed oronasally is very high relative to its cell culture infective dose titer. This also could explain why pig 11 may not have been infected by subsequent contact with its penmates even though at least two of its penmates probably shed virus (Table 1).

There was general agreement between the results of VN and RIP tests in that both identified a primary immune response of 11 of the 12 exposed pigs. Both tests also identified the anamnestic response of pig 1 after treatment with dexamethasone and the reactivation of latent virus (Table 3 and Fig. 1). They differed quantitatively, however, in that there was no general increase in VN antibody titer between the fourth and tenth weeks postexposure, whereas, serum precipitating activity increased markedly for many of the pigs during the same interval. Notice in particular the patterns for pigs exposed to vaccine virus B (Fig. 1).

Our inclusion of RIP in the study was based on previous findings [18, 20] that in some cases it provided indirect evidence for virus reactivation even when virus was not isolated and the serum VN titer was unchanged. A high degree

of sensitivity seemed especially important in working with attenuated viruses for which reactivation and subsequent replication might be minimal. Although the typical pattern associated with reactivation [18, 20] was observed only for pig 1, notice that there was a slight additional increase in serum precipitating activity between the tenth and thirteenth week postexposure for pigs 2, 7, and 8 and perhaps for pigs 5 and 6 (Fig. 1). This change may reflect no more than a continuation of the primary immune response. However, at this point its basis is unclear; especially since we would expect the humoral immune response to reach its maximum sometime within 10 weeks after exposure to virus. An argument for the possibility that it resulted from a brief interval of virus reactivation is weakened, but not entirely excluded, by the lack of a similar change for pig 3 from which reactivated virus was isolated.

Although we only obtained clear evidence for reactivation of virus A, the few pigs per treatment group prevent any meaningful comparison in this regard among the three viruses used in this study. As investigations continue, we may find that most, if not all, attenuated strains of PRV are able to establish latency. Nevertheless, it is conceivable that strains that replicate most extensively in pigs are those most likely to be involved in reactivation; at least in sufficient magnitude to be detected by virus isolation.

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